Stem Cell Reports, Volume *4*

Supplemental Information

Early Depletion of Primordial Germ Cells in Zebrafish Promotes Testis Formation

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Supplemental Figures

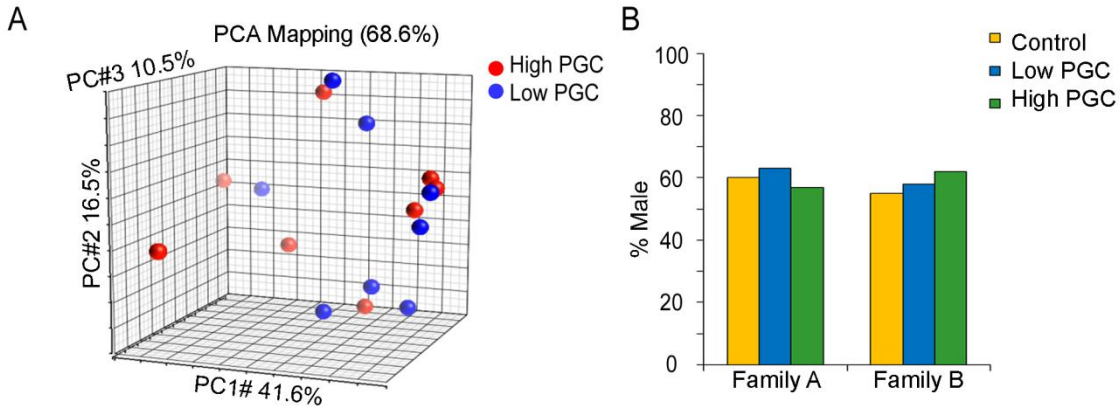

Figure S1: PGC counts at 28-32 hpf in uninjected individuals are not predictive of sexual development, as determined by later trunk-based gene expression profiles and adult sex ratios. Related to Figure 2. (A) Uninjected individuals from the *Tg(vasa:vasa-EGFP)* line were sorted to low and high PGC counts at 28–32 hpf. Eight trunk samples each from the above groups were collected at 22 dpf and analyzed by a qRT-PCR assay using a panel containing 90 pairs of primers designed for 70 genes. A fully overlapping set of points from the 'early high' and 'early low' PGC samples on the PCA plot can be observed. (B) Individuals from two *Tg(vasa:vasa-EGFP)* families (Family A & B) were sorted to high and low PGC group at 28–32 hpf and were grown to 46 dpf to evaluate their sex ratio. The sex ratio of both sorted groups were similar to those of unsorted controls in both families (Family A, n = 163, 78, 72 and Family B, n = 158, 58, 62 for control, low PGC and high PGC, respectively).

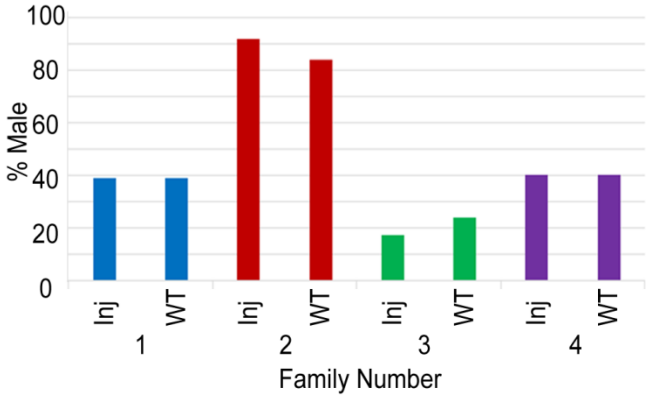

Figure S2: Microinjection with a control morpholino at zygotic stage does not change the adult sex ratio of zebrafish. Related to Figure 2. Zygotes from four different pairs of zebrafish (families #1-4) from the *Tg(vasa:vasa-EGFP)* line were injected with control MO (80 pg) targeted to the human beta-globin intron. The injected (Inj; without prior screening of PGC numbers; n = 240, 36, 35, 126) and uninjected (WT; n = 194, 183, 139, 139) individuals were grown to adulthood for sexual phenotype evaluation. The results showed that the difference between the sex ratios of the injected and the uninjected WT siblings was not statistically significant, suggesting that any effect caused by injection was minimal and random.

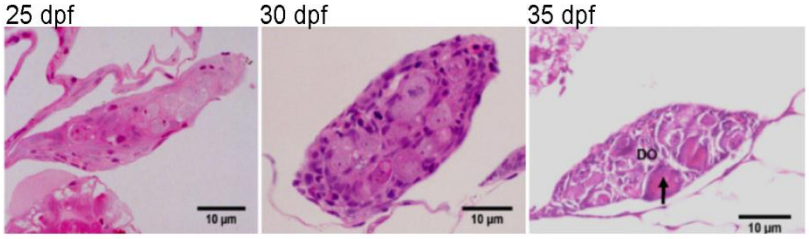

Figure S3: The gonads with heavily depleted PGC number undergo the "juvenile ovary to testis" transformation process. Related to Figure 6. To

investigate whether the development of zebrafish gonads with heavily depleted PGCs was different from that of the wild type, we performed histological analysis between 25 - 35 dpf. The results showed that the juvenile ovary stage occurred in the low PGC number (1-7) zebrafish as was observed in the uninjected control. The gonads with severely depleted PGCs had fewer oocytes but more stromal cells at 25 dpf, and the presence of degenerative oocytes (DO) was detected at 35 dpf. Scale bar: 10 µm.

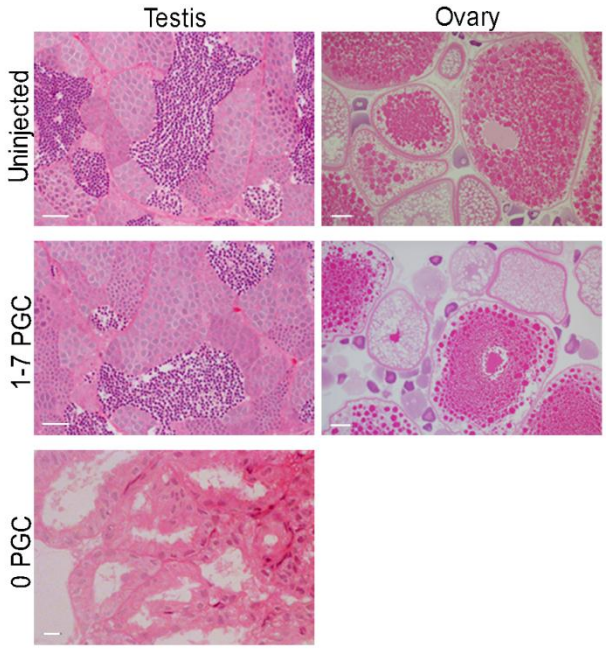

Figure S4: Adult gonads derived from zebrafish with severely depleted PGC number develop similarly to those of the uninjected control. Related to Figure 6. Histological analysis of adult gonads of manipulated zebrafish showed that the structure of both adult testes and ovaries that developed from group I embryos (1-7 PGCs) were similar to those of gonads from group IV (uninjected individuals). Primary and secondary spermatocytes and round spermatids were observed in the testis, while the ovary contained all stages of oocytes. Both adult males and females from group I were sexually active and fertile. In contrast,

zebrafish devoid of PGCs developed testicular structure without having germ cells. Scale bar: 20 μm (testis); 100 μm (ovary).

Supplemental Tables

Table S1: The survival rate of germline chimeras generated by BdT or SPT at different developmental stages. Related to Figure 3.

Method	Experimental group	No. of embryos	No. of normal embryos (%)	PGCs at genital ridge (%)	No. of fish for sexing (%)
SPT	Chimeras	141	87 (61.7)	18 (20.7)	12 (66.7)
	MO	101	89 (88.1)	N/A	60
	Control	85	81 (95.3)	N/A	63
BdT	Chimeras	527	393 (74.6)	372 (94.7*)	97 (26.1)
	MO	197	173 (87.8)	N/A	99
	Control	116	96 (82.8)	N/A	73
Total	Chimeras	608	480 (79.0)	390 (81.3)	109 (28.0)
	MO	298	260 (87.3)	N/A	159
	Control	201	177 (88.1)	N/A	136

* In BdT chimeras, the donor PGCs were present at the gonadal region in most (94.7%) of the host embryos. The rest were lost during migration and ended up at other locations (e.g., the head or the tail region). In contrast to the high success rate of the early germline chimeras, only 26.1% of BdT chimeras grew up to adulthood for the evaluation of sexual phenotypes.

Table S4 : Functional classification of a selected set of genes differentially expressed between WT and PGC-depleted morphants at 14 and 22 dpf. Related to Figure 4.

Developmental stage	Function	Gene symbols*
14dpf		
	Immune	*ccl1, cd209, cyba, irf8, loc799208, zgc:153219*
	Meiosis	*dazl, hormad1, insb, smc1, sycp2*
	Stem/Germ cells	*piwi1, piwi2, tdrd1, tdrd5, tdrd6, tdrd9*
	Cell cycle	*aurka, ccna2, cdc7, chtf18, mcm7, nuasp1*
	Translation	*eif4e1b, mex3b*
	Ovary marker	*org*
	Testis marker	*loc100149647, rnf17*
	Transcription	*snapc1a, tbpl2*
22dpf**		
	Cell death	*baxa, birc5b, bokb, card14, pdcd2, sept4a, siva1, tigara*
	Cell cycle	*btg4, ccna1, ccnb2, ccnd3, ccnh, cdc25, cdca9, fbxo43, mos, sfr1, spdya, wee2*
	Germ/Stem cells	*buc, dnd, gtsf1, henmt1,lhx8a, lin28a, nanog, nanos3,nfr,piwi1, piwi2, pou5f1, rnf17, tdrd1, tdrd12, tdrd5, tdrd6, tdrd7, tdrd9, vasa*
	Meiosis	*dazl, dmc1, hormad1, m1ap1, mei4, meiob, rad21l1, smc1b, spo11, sycp1, sycp2, sycp3, zte38*
	Insulin	*igf3, insb, lmx1a, rfx6*
	TGF-β	*bmp15, dab2, dvr1, foxh1, gdf2, gdf9, gsdf, smad3a, tgfbrap1*
	Retinoic acid	*rbp1b, rdh10b, retsatl, stra6*
	Wnt	*cby1, lgr6, pias4b, wnt8-2*
	Translation/ RNA binding	*caprin2, ddx31, ddx41, eif4e1b, elav2, lsm14b, lsm6, pabpc1l, rbm46*
	Ovary/Testis marker	*figla, foxl2, loc567678, odf3b, org,stk31, zar1, zp2, zp2.2, zp2.5, zp2l1,zp2l2,zp3, zpcx*
	Transcription factor	*ccdc106, dlx6a, drap1, foxa, klf5a, otx1b, pbx2, ripply2, six4a, sox19b, sox8, wu:fi69e09, zglp1*
	Hormone	*cyp17a2, cyp19a1a, fshr, gnrhr2, gnrhr3, gnrhr4, gphb5, hsd3b1, kiss2, pth2r, tshb*
	Immune	*ccl1, cd36, crfb12, il7r, irg1l, irgf1, isg15, ptges, tlr18, tlr3*
	Chromatin	*chtopb, h1m, habp4, npm2, setd8b, suv39h1a*
	Notch	*adam10a, dlk2, jaglb*
	Growth factor	*fgf14, fgf18a, fgf20b*
	Putative male marker	*adam19b, co355608, cyb5r1, klf5a, lin7a, loc100329403, metrnl, pi15, rtn4b, si:dkeyp-66d1.7*

* Genes in black were over-expressed in WT samples, and those in red were up-regulated in the PGC-depleted group at 14 dpf.

******For 22 dpf**,** the gene list was mainly compiled from comparisons between "immature females" vs. a subset of WT and PGC-depleted transforming males (cluster 1 vs. clusters 4&5), "immature females" vs. PGC-depleted transforming males (cluster 1 vs. cluster 5), and among transforming WT individuals (cluster 2 vs. clusters 3&4). Genes in black were up-regulated in the pro-female group, while genes in red were over-expressed in the PGC-depleted or in the pro-male group.

Table S7: Comparative expression analysis of gonad-containing and gonad-less trunks shows that most genes showing differential expression are gonad-enhanced. Related to Figure 4.

	Relative expression			**Relative expression**		
	14 dpf		**Residual**	**22 dpf**		**Residual**
Genes	**(+)gonad**	**(-)gonad**	**expression(%)**	**(+)gonad**	**(-)gonad**	**expression(%)**
birc5b	1.98	0.02	1.09	1.97	0.03	1.64
bokb	1.84	0.16	8.62	1.69	0.31	18.12
btg4	1.88	0.12	6.55	1.97	0.03	1.49
ccdc106	1.83	0.17	9.04	1.83	0.17	9.49
cyp11a1	1.94	0.06	3.33	1.69	0.31	18.28
cyp17a2#	1.98	0.02	0.94	1.55	0.45	29.18
cyp19a1a	N/A	N/A	N/A	1.57	0.24	15.30
dab	1.97	0.03	1.62	N/A	N/A	N/A
dazl	1.99	0.01	0.51	1.92	0.08	4.38
dnd	1.98	0.02	0.89	1.90	0.10	5.26
dram	1.83	0.17	9.43	1.65	0.35	21.57
dvr1	1.72	0.28	16.47	1.81	0.19	10.43
elavl2	1.75	0.00	0.22	1.66	0.09	5.40
*fgf14**	1.59	0.41	26.01	N/A	N/A	N/A
fgf18a	1.75	0.25	14.06	1.61	0.39	23.86
fgf20b	1.78	0.22	12.56	N/A	N/A	N/A
figla	N/A	N/A	N/A	1.75	0.00	0.16
foxh1	1.75	0.25	14.61	1.64	0.36	22.16
foxl2	1.93	0.07	3.58	1.69	0.31	18.65
fshr	1.95	0.05	2.32	1.49	0.26	17.33
gdf2	1.75	0.25	14.13	1.71	0.29	16.66
gdf9	1.96	0.04	2.19	1.92	0.08	4.34
gnrhr2	1.85	0.15	8.06	1.70	0.30	17.84
gnrhr3#	1.78	0.22	12.35	1.53	0.47	31.04
gsdf	1.64	0.36	21.61	1.97	0.03	1.55
gsdf2	1.94	0.06	2.83	N/A	N/A	N/A
hormad1	1.99	0.01	0.46	1.90	0.10	5.04
ihx8a	1.90	0.10	5.32	1.90	0.10	5.02
il7r#	1.79	0.21	11.5	1.55	0.45	29.21
insb	1.91	0.09	4.71	1.85	0.15	8.25
*irf8**	1.49	0.51	34.20	N/A	N/A	N/A
kiss2	1.84	0.16	8.50	N/A	N/A	N/A
*klf3**	1.49	0.51	34.67	N/A	N/A	N/A

lin28a	1.86	0.14	7.81	1.85	0.15	7.84
metrnl	1.74	0.26	14.81	1.61	0.39	24.02
mos	1.85	0.15	8.38	1.83	0.17	9.18
nanog	1.64	0.04	2.56	2.50	0.00	0.05
nanos3	1.74	0.26	15.26	1.84	0.16	8.46
nfr	1.69	0.31	18.16	1.74	0.26	15.21
npm	1.74	0.26	14.83	1.82	0.18	9.77
odf3b	1.93	0.07	3.54	1.83	0.17	9.11
org	1.93	0.07	3.64	1.96	0.04	1.92
pdcd2	1.74	0.26	15.14	1.60	0.40	24.98
pias4b	1.93	0.07	3.68	1.88	0.12	6.17
piwi1	N/A	N/A	N/A	1.88	0.12	6.15
piwi2	1.95	0.05	2.60	1.84	0.16	8.56
pou5f1	1.91	0.09	4.56	1.93	0.07	3.57
rbp1b	1.95	0.05	2.42	1.93	0.07	3.63
rdh10b#*	1.51	0.49	32.80	1.51	0.49	32.28
retsatl	2.00	0.00	0.23	1.74	0.01	0.34
rnf17	1.93	0.07	3.76	1.80	0.20	11.31
sept4a	1.71	0.29	16.68	1.82	0.18	9.67
smc1b	1.81	0.19	10.59	1.41	0.09	6.75
sox19b	1.84	0.16	8.89	1.62	0.38	23.67
sox9a	1.83	0.17	9.38	N/A	N/A	N/A
spo11	1.97	0.03	1.50	1.90	0.10	5.10
star#	1.95	0.05	2.34	1.51	0.49	32.66
stra6	1.90	0.10	5.39	1.88	0.12	6.31
sycp3l	1.95	0.05	2.33	1.88	0.12	6.20
tdrd9	1.81	0.19	10.25	1.78	0.22	12.27
wnt4a	1.83	0.17	9.11	1.76	0.24	13.71
wnt8	1.25	0.04	3.12	1.98	0.02	0.94
zp2	2.02	0.23	11.52	1.99	0.01	0.29
Muscle associated genes (positive controls)						
acta1	1.22	1.04	84.63	1.02	0.97	94.94
mylpfa	1.27	0.93	73.39	1.18	0.87	73.74
myostatin	1.22	1.04	85.77	0.93	1.05	113.66
pax3	1.07	0.93	86.18	1.22	0.78	63.85
pax7	0.93	1.07	116.13	1.10	0.86	78.53

* - > 25% residual expression in trunk (without gonad) samples collected at 14 dpf.

- > 25% residual expression in trunk (without gonad) samples collected at 22 dpf.

Supplemental Experimental Procedures

Manipulation of PGC number in zebrafish embryos

We used individuals from the *Tg*(*vasa:vasa-EGFP*) transgenic zebrafish line for investigating the role of PGCs during gonadal development. We manipulated the PGC number in zebrafish embryos by using *dead end* (*dnd*) morpholino (*dnd*-MO), as described previously (Weidinger et al., 2003). In order to achieve partially depleted PGC numbers, we first titrated concentrations of *dnd*-MO, which were microinjected into embryos at one cell stage using a PLI-100 Pico-Injector (Harvard Apparatus). The optimal concentration of *dnd*-MO required to generate embryos with various numbers of PGCs was determined to be 80-100 pg per embryo. In the *Tg(vasa:vasa-EGFP)* transgenic line, EGFP is maternally deposited in the embryos and the product of zygotic expression localizes only into the germ cells. We counted the PGC number via monitoring the GFP signal under a dissecting microscope (Leica) equipped with a fluorescent attachment (MAA-03/B; BLS Ltd, Budapest, Hungary) over the time period of 52-86 hpf. To facilitate the PGC counting, embryos were depigmented by treating them with 1-Phenyl 2-thiourea (PTU) at 24hpf.

Mass cross approach

Altogether, around 6,000 embryos from six different batches of eggs produced by mass crosses were injected with 100 pg *dnd*-MO per embryo, which yielded embryos with various numbers of PGCs. The *dnd* morphants were broadly categorized into four different groups: group I (1-7 PGCs), group II (8-15 PGCs), group III (>15 PGCs), group IV (uninjected control) and embryos with no PGC were designated as group V, which was used as control. The *dnd* morphants and uninjected embryos (control) were grown to 3 months post-fertilization (mpf) for evaluation of their sexual phenotype by (i) the differential expression of EGFP in the gonads, whereby the presence of a high level of EGFP signal was shown

earlier to correlate with ovarian differentiation, (ii) external phenotype, and (iii) occasional dissection for verification.

Pairwise cross approach

In addition, we performed pairwise crosses with a similar experimental setup as described above. Briefly, ca. 1000 embryos of the *Tg(vasa:vasa-EGFP)* zebrafish line of mating pairs with known offspring sex ratios were microinjected with *dnd*-MO (total 4 replicated experiments). The ideal concentration of *dnd* MO for generating a wide range of PGC number was 80 pg per embryo after titration. Injected embryos (without PTU treatment) were grouped based on the number of PGCs identified between 24-32 hpf under the compound epifluorescence microscope. Several groups of zebrafish including no PGC (0), low PGC number (1-6, 7-10), medium PGC number (11-20), and high PGC number (> 20) were generated. The sex ratio of *dnd* morphants was evaluated at adulthood (3 mpf).

Production of germline chimeras

To produce germline chimeras, a single PGC transplantation (SPT) and blastoderm transplantation (BdT) were performed. In general, the SPT method yields germline chimeras that possess a single donor-derived PGC with no somatic cell contamination, while BdT method was more useful to produce germline chimeras that possess more than one PGC. Detailed procedures for SPT and BdT were described elsewhere (Saito et al., 2010; Yamaha et al., 2001). Briefly, a GFP-labeled PGC was derived from a 10-15 somite embryo injected with GFP-*nos3* 3'UTR mRNA, and was subsequently transplanted into the host blastula. The resultant chimera possessed a single donor derived PGC and named as SPT chimera. For BdT chimeras, the *Tg*(*vasa:DsRed2-vasa*);*Tg*(*bactin:EGFP*) double transgenic zebrafish line was used as the donor. Therefore, the host and donor cells were easily distinguished under fluorescent microscope since donor cells started expressing GFP around the early blastula stage under driven by β-actin promoter. During the early to mid-blastula stage,

the upper half of the blastoderm was cut and removed from the host embryo. Then, the whole donor blastoderm was cut and transplanted onto the host blastula. Consequently, the donor blastoderm adhered to the recipient and became involved in its development in a few hours (Figure 3C). Both the procedure of creating BdT chimeras and the process of mixing up donor and host blastoderm were shown in Movie S1 and Movie S2.

To distinguish offspring by body color, wild type zebrafish were used as the donor and golden zebrafish as the host. All host embryos were received *dnd* -MO to deplete endogenous PGCs completely (Ciruna et al., 2002; Weidinger et al., 2003). Confirmation of complete depletion was checked by microscopic observation of empty gonads in the *dnd* morphants. The number of PGCs in each chimera was counted under the fluorescent inverted microscope (Leica DMI6000B) at the prim-5 stage. The gonads of BdT chimeras were examined to determine whether the germline chimeras only possessed RFP (+) germ cells. Each chimera was kept separately until identification of the two sexes based on phenotypic signs and/or dissection of their gonads became possible. Images of the embryos were obtained using a Leica MZ16F fluorescence stereomicroscope equipped with a digital camera (Leica DFC300FX). The gonadal phenotype of individuals developing from the germline chimeras was determined at adult by analyzing their secondary sex characteristics.

Confocal microscopy

The larval trunks embedded in 1.5% low melting agarose were imaged on a Leica SP5 inverted confocal microscope equipped with a HCX PL APO 40x/1.25 N.A. oil objective lens. Samples were illuminated with 488 nm to excite the GFP-tagged protein and with 561 nm to excite the autofluorescence of pigmented tissues, in order to remove false positives from the analysis. Due to large variations in GFP intensities and sample depth, laser power was adjusted to optimize contrast for individual samples. Confocal Z-stacks were imaged every

0.8 µm from 40-120 µm, depending on the sample thickness and the position of PGCs within the tissue.

RNA sample preparation and processing

RNA samples were extracted using Ambion RNAqueous-Micro Kit (Life Technologies). RNA quality was assessed by the Agilent 2100 Bioanalyzer with the RNA 6000 Pico LabChip (Agilent Technologies). Only samples with RIN value > 8 were used for subsequent microarray analysis. RNA concentration was quantified using Qubit 1.0 Fluorometer with RNA Assay Kit (Life Technologies).
Due to small amounts of RNA isolated from individual sample, whole transcriptome amplification (WTA) was performed prior to microarray hybridization. A total of 15 ng RNA was used for amplification with Ovation RNA Amplification System V2 (NuGEN). The size distribution of the amplified double stranded cDNA samples were checked by Agilent 2100 Bioanalyzer with RNA 6000 Nano Labchip (Agilent Technologies) to ensure fragment size uniformity among the samples.

One µg of the double stranded cDNA from each sample was labeled using NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen), and hybridization was carried out according to the manufacturer's instructions. After overnight hybridization (16-20 hours), the microarray chip was washed with NimbleGen Wash Buffer Kit (Roche NimbleGen). The array was then scanned at 5 µm resolution with Axon GenePix 4000B Microarray scanner (Molecular Devices).

Microarray data analysis

The microarray data was collected and analyzed according to the MIAME standards (Brazma et al., 2001). The raw fluorescent intensity data were retrieved from the scanned images by NimbleScan version 2.6 (Roche NimbleGen) according to the manual. Robust multi-array average (Irizarry et al., 2003), quantile normalization (Bolstad et al., 2003) and background correction were applied as implemented in NimbleScan to generate the Pair files. The Pair

files were then imported into the Partek Genomics Suite version 6.6 (Partek Incorporated) for further analysis. The DE transcripts between the PGC-depleted (1-9) group and WT group were identified as significant at least 2-fold, at p <0.05 (false discovery rate-adjusted).

Quantitative RT-PCR

Specific target amplification was carried out on the cDNA (from total RNA after WTA) using TaqMan PreAmp Master Mix (Applied Biosystems), and the products were loaded onto the Fluidigm's Dynamic Array Integrated Fluidic Circuits (IFC) according to Fluidigm's EvaGreen DNA Binding Dye protocols. Six PGC-depleted morphants and ten WT samples were selected for analysis; triplicates were for each sample.

Histology

The tissues were fixed in 4% paraformaldehyde at 4˚C overnight. After dehydration, samples were embedded in HistoResin (Leica). Serial sections of 5 um were cut by microtome (Leica), dried on slides at 42˚C overnight, stained with hematoxylin and eosin (H&E), mounted in Permount (Fisher), and imaged with phase contrast with a 100x/1.4 N.A. oil objective lens.

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